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DETECTION OF MURINE TYPHUS INFECTED FLEAS WITH AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract. An enzyme-linked immunosorbent assay (ELISA) for the detection of *Rickettsia typhi* antigen in homogenates of pooled or individual laboratory infected fleas is described. The assay uses a double sandwich technique, employing a pool of monoclonal antibodies to capture the antigen and a hyperimmune rabbit serum for antigen detection. Using pools of *R. typhi* infected *Xenopsylla cheopis*, *Ctenocephalides felis*, and *Leptopsylla segnis*, the sensitivity of the ELISA was compared with direct fluorescent antibody examination of individual fleas for rickettsiae and with rickettsial titers determined by plaque enumeration on primary chicken embryo fibroblasts (PFU). Pooled samples with <4 PFU of viable rickettsiae gave ELISA results which were not significantly above background. Both ELISA OD and ELISA titer (last dilution giving an OD that was 2 SD above the control) of a 1:10 dilution of homogenate (4 fleas/ml) were linearly related to rickettsial titer up to $10^{6.8}$ PFU/sample. Multiple freeze-thaws of pools of infected fleas led to a rapid loss of ELISA sensitivity. ELISA assays on single fleas demonstrated large individual variability in rickettsial content. This was independent of the number of days postinfectious feeding or the mean number of PFU/flea ($10^{1.7-6.9}$) found for pooled fleas in the same cohort. The sensitivity and ease of performance of ELISA should make it usable under field conditions.

Rickettsiae are transmitted by diverse hematophagous arthropods. Non-human reservoirs of these diseases include rats, mice, flying squirrels, and many other mammals.[1,2] Current field survey methodology is cumbersome.[3] The availability of an assay that could directly detect the infectious agent and be adapted to testing large numbers of specimens would be an invaluable aid to field studies.

The production of monoclonal antibodies (Mabs) specific for major surface antigens of rickettsial species[4–6] has made it possible to use sensitive assays to detect and to differentiate rickettsial species in arthropod vectors. The enzyme-linked immunosorbent assay (ELISA) is a suitable candidate method for the detection of antigen in test samples and has distinct advantages over immunofluorescence or other methods.[7] The cost effectiveness of the ELISA over isolation methods has been established for arboviruses.[8] Numerous laboratory studies of specific antigen capture ELISAs have been described for the detection of different sporozoites[9,10] and viruses in mosquitoes.[11–14] Similarly, an ELISA was described for detection of Crimean-Congo hemorrhagic fever virus in naturally infected ticks.[15] Some mosquito assays have proved satisfactory for field survey applications.[16,17] No antigen capture ELISA has been described for any of the rickettsiae. We describe a double sandwich, antigen capture ELISA for the detection of *Rickettsia typhi* antigen in laboratory infected fleas. The sensitivity and reliability of this assay make it a good candidate for further evaluation in field studies.

MATERIALS AND METHODS

Antigens

Rickettsia typhi strain AZ332 (Ethiopian)[18] was used to infect fleas. *R. typhi* infected fleas were obtained by feeding them on rickettsemic laboratory rats. Flea samples were collected at day 0, and every other day thereafter, and stored at −70°C. Although 3 different species of fleas were used for ELISA assay (*Ctenocephalides felis*, *Leptopsylla segnis*, and *Xenopsylla cheopis*), most

experiments have utilized *X. cheopis*. The flea rearing procedures, methods of infecting and handling of inflected fleas were similar to those described previously.[18-22]

Detection of rickettsiae in fleas

Rickettsial infection in individual fleas collected at various intervals after infectious feeding was determined by direct immunofluorescent antibody test (DFA).[19] DFA was performed by staining flea gut smears using FITC-labeled anti-*R. typhi* (convalescent) guinea pig serum. To quantify the live rickettsiae present in fleas, the chicken embryo cell plaque assay method was used on homogenates of pooled flea samples.[19-21] Fleas were surface sterilized and were triturated in brain heart infusion broth (BHI) in pools of 10 fleas/2.5 ml. The homogenates were then stored frozen at −70°C. For rickettsial titration of each homogenate, 5 samples of 0.2 ml each undiluted and 6 serial decimal dilutions of each flea homogenates were inoculated onto monolayer tissue cultures of chicken embryo fibroblasts.[20-22] Controls included rickettsial seed with known plaque forming unit (PFU) values and uninfected fleas.

Monoclonal antibodies

Mouse hybridoma cell lines were prepared by conventional selection techniques following polyethylene glycol mediated cell fusion. SP2/0-Ag14 plasmacytoma cells were fused with spleen cells from BALB/c or NMRI mice which had been hyperimmunized with either Renografin density gradient purified *R. typhi* strain Wilmington, *R. prowazekii* strains Breinl or Madrid E, or the purified 120 kDa surface protein antigens (SPAs) of those species.[23-25] The monoclonal antibodies (Mabs) were selected by ELISA assay, Western blotting, and biological assays. The antibodies were used as undiluted culture supernatants derived from limiting dilution cloned hybridomas grown in RPMI 1640 with 10% fetal bovine serum. The supernatants were stored at 4°C with 0.02% Thimerosal as preservative.

Polyclonal antibodies and enzyme conjugate

Rabbit anti-*R. typhi* hyperimmune serum was made by immunizing New Zealand white rabbits with 6 doses at 21 week intervals of 0.5 mg of Renografin purified *R. typhi* cells which had been partially depleted of SPA by 2 extractions with distilled water.[24] The antigen was emulsified 1:1 with Freund's incomplete adjuvant and presented in multiple small volumes im and sc. The final serum was obtained 10 days after the last immunization and had an ELISA titer[25] $>10^6$ against native SPA and $>10^5$ against heat denatured SPA. By Western blotting, the serum also contained antibodies in high titer against lipopolysaccharide and 6 other protein antigens. Affinity chromatography purified goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase were obtained from Cooper Biomedica, Inc., Malvern, PA. Optimum concentrations of the rabbit and goat antibodies needed for maximum ELISA sensitivity were determined by block titration of each reagent using preparations of Renografin purified *R. typhi* whole cells which had been frozen and thawed once in distilled water.

Double sandwich antigen capture ELISA

We coated 96-well, round-bottom, Nunc Immulon II polystyrene plates (PGC Scientific, Gaithersburg, MD) with 100 μl/well of goat anti-mouse IgG diluted 1:3,000 in PBS, pH 7.0. The plates were incubated at 4°C overnight in a moist chamber and then washed 3 times with PBS plus 0.05% Tween 20, 1% dextran sulfate, and 5% horse serum (PBSX). We added 100 μl/well PBSX with 5% gelatin as a blocking reagent, and then incubated the plates at 37°C for 30 min. Following 3 successive PBSX washes, the following were added successively to each plate at 100 μl/well and incubated for 1 hr at 37°C: a pool of anti-typhus Mabs (Table 1); test specimens (flea homogenates [10 fleas/2.5 ml BHI] heated at 60°C for 30 min to inactivate any rickettsiae present) diluted 1:10 and serially 2-fold to 1:1,280 in PBSX, plus 10 replicates each of positive (purified rickettsial cells) and negative (diluent) controls; rabbit anti-*R. typhi* antiserum diluted 1:2,000 in PBSX; and horseradish peroxidase conjugated goat anti-rabbit IgG antibody diluted 1:500 in PBSX. After 3 final washes in PBSX, 100 μl/well of 2,2'-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS) substrate from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD) was added for 30 min at 37°C. Absorbance values were read spectrophotomet-

TABLE 1

Characteristics of anti-SPA monoclonal antibodies used in double sandwich antigen capture ELISA for Rickettsia typhi *antigen*

Antibody	Typhus specificity*	Immunoglobulin subclass	Heat sensitive epitope	Toxin neutralizing	Sensitivity (ng/assay)
1. T65-1G9.3	T only	KG2a	+	+	10
2. T66-1E8.1	T only	KG1	−	−	100
3. TP29-6C3.2	T, PB, PE	KG1	+	−	10
4. P50-2B8.1	T, PB, PE	KG1	−	−	10
Pool of 1–4					<1
5. T11-3C7.2	T only	KG2a	+	+	100
6. T65-1G2.2	T only	KG1	−	−	100
7. T66-2D3.1	T, PB, PE	KG2b	−	−	1,000
8. T66-1D12.1	T, PB, C	KG3	−	−	1,000
9. P51-4D8.1	T, PB, PE, C	KG2a	−	−	1,000
10. P53-3D1.1	T, PB, PE, C	KG1	−	−	1,000
Pool of 1–10					1

* T = *R. typhi* Wilmington, PB = *R. prowazekii* Breinl, PE = *R. prowazekii* Madrid E, C = *R. canada* McKiel.

rially against an air blank at 414 nm using a Multiscan ELISA reader (Flow Laboratories, Inc., McLean, VA). Optical densities of test specimen dilutions which exceeded the mean plus 2 SD of the OD of the negative flea controls were considered positive.

Single flea ELISA modification

Fleas were triturated in 100 μl BHI and inactivated for 30 min at 60°C. The full volume was added to 1 assay well, taking care to exclude the exoskeleton material. Initial experiments with selected homogenates of flea pools demonstrated that a modified wash diluent, PBS with 0.05% Tween 20 (PBST) and a blocking buffer PBST with 0.5% boiled casein, gave results comparable or better to PBSX and PBSX with gelatin, respectively. Consequently, all the single flea experiments utilized the simpler PBST based diluents rather than PBSX in the ELISA described above. Absorbance values for single fleas were obtained with a Model 2550 plate reader (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Development of ELISA

Ten well-characterized Mabs, differing in their immunoglobulin subclass, epitope recognition of the 120 kDa SPA proteins of typhus group rickettsiae (species specificity, heat sensitivity), and their ability to neutralize intravenous rickettsial toxicity for mice, were evaluated individually and as pools for their usefulness in the double sandwich antigen capture assay (Table 1). Detection sensitivities for purified whole cell preparations of *R. typhi*, which had been frozen and thawed in distilled water, varied between 10–1,000 ng protein/100 μl assay volume. However, a pool of all 10 antibodies and a pool of the 4 most sensitive individual antibodies both increased the detection sensitivity 10-fold to ≤1 ng protein/100 μl assay volume. To simplify reproduction of the pool of antibodies, the pool of antibodies 1–4 was used in all subsequent experiments.

Application of ELISA to homogenates of pooled fleas

Eight pools of infected *X. cheopis* with known PFU/flea titers were evaluated in the ELISA capture assay to establish relative sensitivities and detection limits (Figs. 1, 2). The ELISA ODs of uninfected flea pool samples differed little from dilu[illegible] controls (not shown). Only one of the posi[illegible]ve fl[illegible] pools (120 PFU/flea) was not positive [illegible] ISA at a 1:10 dilution, while pools with 250 [illegible]FU/flea and 450 PFU/flea were readily detected. Using the regression line and, as minimum cutoff, positivity at a 1:10 pool dilution, the minimum detection limit is about 100 PFU/flea. This corresponds to about 4 PFU in the 100 μl assay volume. A linear response of ELISA titer to the pool PFU measurements was also observed at up to $10^{6.8}$ PFU/flea ($r = 0.97$). To eliminate manipulations and reduce the time involved in estimating the extent of rickettsemia by assays based on endpoint dilution of flea extracts, the ELISA OD of the 1:10 dilutions of

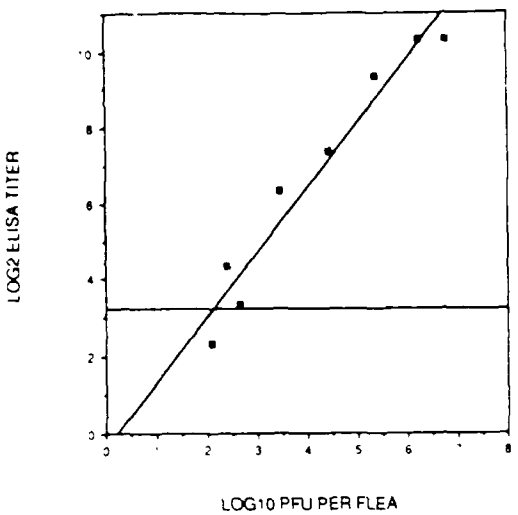

FIGURE 1. Comparison of ELISA titers and PFU/flea determinations on 8 pools of infected *X. cheopis* fleas. Regression line of data is $\log_2$ ELISA titer = $-0.379 + 1.697 \log_{10}$ PFU flea. Horizontal line indicates the limit of positive ELISA determinations on pools at a 1:10 dilution of the pools.

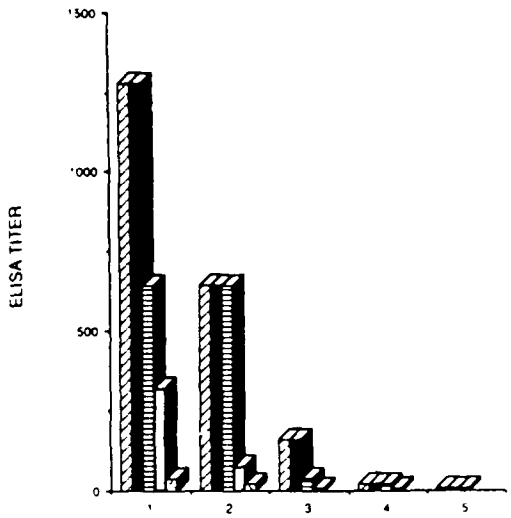

FIGURE 3. Effect of freeze-thaw treatment on ELISA titers of 5 *X. cheopis* flea pools.

homogenate were also compared to PFU/flea titer (Fig. 2). There was a linear relationship ($r = 0.97$) up to $10^{6.8}$ PFU/flea where the OD reached 2.5, the limit of the scale. The detection limits were approximately 100 PFU/flea or 4 PFU in the 100 μl test sample. Although flea pools occasionally can exceed the high limit, the average rickettsial burden of most flea pools can be estimated simply from the ELISA OD at a fixed dilution.

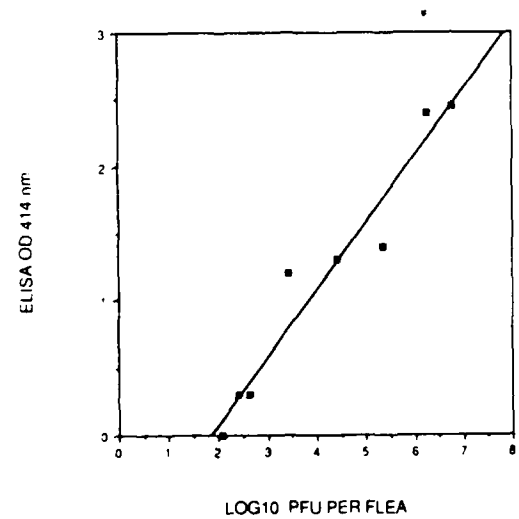

FIGURE 2. Comparison of ELISA ODs and PFU/flea determinations on the same 8 pools of infected *X. cheopis* fleas as Fig. 1. Regression line of data is ELISA OD = $-0.917 + 4.995 \log_{10}$ PFU/flea. ELISA determinations were all done on flea pools diluted 1:10.

Sample treatment effects on ELISA sensitivity

Repeat ELISA measurements utilizing the same undiluted flea pool homogenates after 1–5 freeze-thaws demonstrated that such treatment causes a pronounced loss of antigenic reactivity (Fig. 3). Since this loss of reactivity did not occur with purified rickettsial antigens, it is likely that flea proteases degraded the SPA protein antigen and that multiple freeze-thaw treatments of homogenized fleas must be avoided.

Application of ELISA to different flea species

To further demonstrate the usefulness of the assay and to examine its limitations, the ELISA was compared to direct immunofluorescent antibody test (DFA) and plaque assay on 1:10 dilutions of 35 pools each of *X. cheopis*, *C. felis*, and *L. segnis* obtained at different times after feeding on rickettsemic rats (Table 2). Only one *X. cheopis* pool was DFA and PFU positive but ELISA negative (7 days post-feeding). This sample was the only case in which the $\log^{10}$ PFU/flea titer (1.7) was below the expected level of

TABLE 2

Comparison of direct fluorescent antibody test, rickettsial cell culture plaque forming units, and antigen capture ELISA for detecting Rickettsia typhi *infection of fleas*

No flea pools	Flea species	Day*	DFA†	Rickettsial titer‡	No ELISA positive pools
5	*X. cheopis*	0–5	0/60	all <0.1	0
3	*X. cheopis*	7–9	20/20	1.7, 2.8, 5.9	2
3	*X. cheopis*	11–13	20/20	4.3, 5.7, 6.5	3
3	*X. cheopis*	15–19	20/20	4.5, 4.9, 6.2	3
3	*X. cheopis*	25–32	15/15	4.7, 5.7, 6.1	3
5	*L. segnis*	0	0/50	all <0.1	0
2	*L. segnis*	5	5/20	2.3, 2.4	2
2	*L. segnis*	7–11	27/30	4.1, 5.8	2
2	*L. segnis*	20–30	10/10	5.2, 6.6	2
3	*C. felis*	0	0/50	all <0.1	0
2	*C. felis*	7–9	20/20	3.7, 4.3	2
2	*C. felis*	13–21	20/20	5.4, 5.7	2

* Days postinfectious feeding.
† Number positive fleas/total fleas by direct fluorescent antibody method
‡ Log_{10} PFU/flea.

assay sensitivity (Fig. 1). None of the 13 control flea pools obtained on days 0–5 post-feeding, in which DFA and PFU measures of rickettsial infection of fleas were negative, gave positive ELISA data. The pools of *L. segnis*, with less than 100% DFA infectivity (25% on day 5 postinfection, 90% on days 7–11 postinfection), were both PFU and ELISA positive. Consequently, even at a greater dilution than that used for PFU determinations, the ELISA exhibited 100% specificity and 93% sensitivity (28/30) (Fig. 1, Table 2) in detecting rickettsial infection of fleas relative to PFU determinations.

Single flea ELISA assay

To determine whether the ELISA could be applied to single fleas, as might be encountered in small random samples, 99 individual *X. cheopis* were assayed by ELISA. Similar cohorts of fleas were assayed directly by DFA or pooled and assayed for PFU (Figs. 4, 5). As had been found with the flea pools, only 1 of 14 infected fleas from cohorts without detectable PFU and negative by DFA (0/15) were positive by ELISA; this flea exceeded the cutoff by only 0.018 OD. The ELISA values of single fleas exhibited considerable variability when plotted against the average log_{10} PFU/flea of pools of fleas from the same cohorts (Fig. 4). The regression between these measures also exhibited a much lower correlation ($r = 0.38$) than that found from similar comparisons made previously on pools (Fig. 2). However, the x intercept of the regression indicated that 5 PFU/flea was the lower limit of sensitivity for the ELISA, a value very similar to the 4 PFU estimated previously with the smaller number of pools (Figs. 1, 2).

The ELISA was also compared with log_{10} PFU/flea by averaging all the data obtained at various days postinfectious feeding (Fig. 5). PFU/flea determinations on pools at the same time points had standard deviations in data not greatly dif-

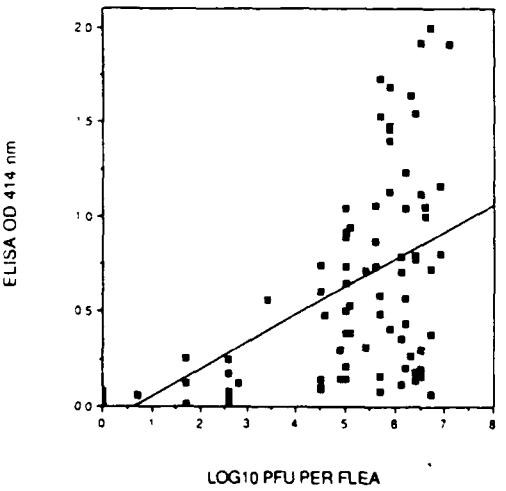

FIGURE 4. Comparison of ELISA OD obtained on 99 single *X. cheopis* fleas with PFU/flea determinations on pooled fleas from the same cohorts. Regression line of data is ELISA OD = $-0.098 + 0.144$ log_{10} PFU/flea. Includes 14 infected fleas (0 days postinfection) with overlapping ELISA values at 0 = log_{10} PFU/flea. Only 1 of these 14 fleas exceeded the OD cutoff for rickettsial positivity (OD = 0.059).

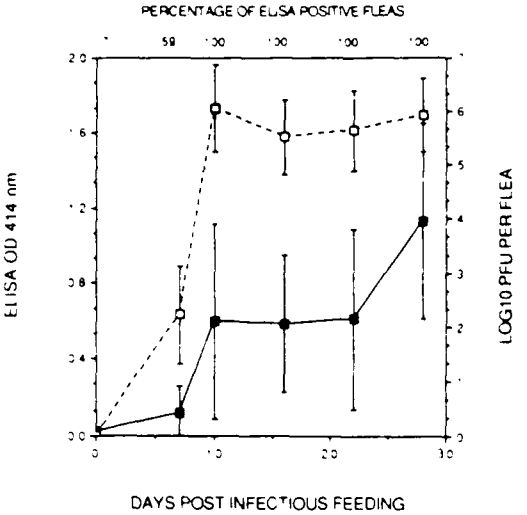

FIGURE 5. Comparison of ELISA OD (■——■) and PFU/flea (□- - -□) determinations on single *X. cheopis* fleas (data of Fig. 4). Error bars are standard deviations of data. Number of determinations (ELISA/PFU) for days 0, 7, 10, 16, 22, and 28 are 14/9, 17/7, 25/10, 17/9, 8/5, and 18/7, respectively. DFA determinations were 0/15 at day 0 and 20/20 at day 10 (positive/total examined).

ferent from the deviations of ELISA ODs obtained with single fleas, thus suggesting that fleas vary considerably in their individual rickettsial burdens. All of the fleas were DFA positive (20/20) by 10 days post-infectious feeding, a time at which all fleas were also ELISA positive (Fig. 5). Consistent with prior findings by DFA,[19-21] all of the fleas remained ELISA positive over the times tested.

DISCUSSION

This study confirms that antigen capture ELISA is suitable for the detection of *R. typhi* antigen in 3 species of fleas. It can readily detect 1 infected flea having 10^4 PFU in a pool of 40 negative fleas homogenized in a 10 ml volume. *X. cheopis*, *L. segnis*, and *C. felis* generally have more than 10^4 PFU/flea by 7–10 days post-infectious feedings until their death up to 40 days later. Indeed, rickettsial titers per flea can exceed 10^6 for *C. felis* and 10^7 for *X. cheopis* and *L. segnis*.[19-22, 26] Even during the log phase of rickettsial growth immediately following infection of the fleas, 7 out of 9 pools of fleas with $< 10^4$ PFU/flea were ELISA positive even though only 0.4% of the pool volume was assayed. The ELISA sensitivity was surprising since calibration of assay sensitivity with purified rickettsiae suggested that only 1 ng protein of rickettsial antigen could be detected. Assuming 100% of the antigen is viable, 1 ng should contain about 10^4 PFU,[27] not the 4 PFU detection limit found for fleas. This suggests that fleas either contain large amounts of non-viable rickettsiae (at least not measurable by either PFU or animal titrations which give comparable results), or that excess 120 kDa SPA antigen is present relative to the amount found in intact purified yolk sac grown rickettsiae, or both. Heterogeneity in flea production of excess antigen might help to account for the great variation encountered in ELISA ODs with single flea samples and for the very large increase in ELISA reactivity relative to PFU determinations in the flea specimens taken 28 days post-infection (Fig. 5).

Examination of single fleas for rickettsial infection by either DFA or rickettsial titration is feasible. However, the DFA technique is extremely laborious, semi-quantitative at best, and does not distinguish viable from non-viable rickettsiae. Similarly, titration of viable rickettsiae in either mice or tissue culture is expensive, requires at least 10 days, is most safely done in a specially designed containment laboratory, and is subject to loss of data from the technically demanding plaquing technique. In contrast, the ELISA capture assay is simple, sensitive, and rapid. Fleas, flea feces, and even gut samples can be assayed without altering the procedure. Although the ELISA, like the DFA, does not distinguish between live or dead rickettsial antigen, it may be advantageous over the DFA for field applications where it may be difficult to maintain flea viability until the DFA assay can be done: samples may be frozen immediately, or even dead fleas may possibly be used. The ELISA is also superior to the DFA in permitting either visual semiquantitation (especially if titrations are employed) or precise, computerizable spectrophotometric quantitation. The ELISA should be suitable for studies of transovarial transmission of rickettsiae since the F1 generation infection rates are low and thus require large flea samplings.[28]

Although both rat and mouse Mabs against other typhus rickettsial antigens, including lipopolysaccharide and other proteins, are available, Mabs against the 120 kDa SPA proteins were selected for several reasons. First, each of

the other antigens are largely group-reactive, being present in *R. typhi*, *R. prowazekii*, and *R. canada*. Indeed, homologous proteins and lipopolysaccharides with epitopes in common with typhus antigens are present in both spotted fever group rickettsiae and other gram negative bacteria. The most restricted epitopes found to date in both typhus and spotted fever group rickettsiae reside on their 120 or 160 kDa SPA proteins. The SPA antigens comprise about 15% of the total cellular protein of these species and are located outside the cell envelope in a regular array (S layer proteins), which is highly accessible to antibody.[4–6] Finally, the typhus SPAs are readily released from rickettsiae by hypotonic shock.[4] Preliminary data suggests that ELISA assay sensitivity can be improved by using water rather than BHI for flea homogenization and by using formalin addition rather than heat for rickettsial inactivation. We anticipate that a simplified assay using a single high-affinity purified species-specific Mab as capture reagent and direct enzyme conjugates of mouse or rat Mabs for detection may distinguish *R. typhi* from *R. prowazekii* in ectoparasite samples. Since suitable specific antibodies are available for *R. prowazekii*, *R. canada*, and various spotted fever rickettsial species,[4–6] it should be possible to detect other rickettsial species in lice, ticks, and mites. Since infectivity titrations of field collected, DFA positive fleas had 10^{3-4} PFU/flea (A. F. Azad, personal communication), it is likely that the capture ELISA will have field applicability.

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